

Methods

Reagents

HA-tagged versions of S3 and S20 in pcDNA3 were provided by Dr Sonia Lain. The Flag tagged L5 and L11 were provided by Dr Karen Vousden. HA-tagged versions of S7 and L7 were made in pcDNA3 using IMAGE clones (3940495 and 4134557 respectively). Sequences were confirmed by automated sequencing. Anti-HA and anti-Flag antibodies were purchased from Babco and Sigma respectively. Rabbit anti-NEDD8 antibody and anti-ubiquitin FK2 mouse antibody was purchased from BIOMOL. Goat anti L11 antibody was purchased from SantaCruz. Anti-APPBP1 mouse antibody was purchased from Abnova and anti- β -gal mouse antibody from Cell Signaling. Mouse anti-SV5 antibody was produced in house. Secondary antibodies were from Amersham. MG132 was purchased from CalBiochem. Human NEDD8 gene was initially cloned in frame in the TAP/pCMV-5 vector. The TAP-NEDD8 fusion gene was then amplified and cloned as an XhoI/XbaI insert in the pEFIRE5-P vector. Hela cells stably expressing TAP-NEDD8 were selected under puromycin (5 μ M).

Isolation of NEDDylated target proteins.

150x10cm dishes of 90% confluent Hela cells stably expressing TAP-NEDD8 were lysed in 2% SDS, 50mM Tris-HCl pH 8.0, 10mM iodoacetamide, 1mM EDTA and protease inhibitors (EDTA-free cocktail tablets, Roche). The extracts were sonicated (4x15sec) and diluted 25x with renaturation buffer containing 50mM Tris-HCl pH8.0, 0.5M NaCl, 1% NP-40, 0.5mM EDTA, 1mM PMSF. The sample was passed over a 1.5ml IgG sepharose column (Amersham Biosciences) previously equilibrated with renaturation buffer. The column was then washed with 5 column volumes (CV) of renaturation buffer and 10 CV of TEV cleavage buffer: 50mM Tris-HCl pH 8.0, 0.5M

NaCl, 1% NP-40, 1mM DTT. After the final wash the column was resuspended in 3ml (final volume) of TEV cleavage buffer, 250µg of TEV protease were added and cleavage was performed overnight at 4°C by gentle rotation. Next day the second purification step was performed, using calmodulin resin (Stratagene). 0.5ml of packed volume of beads were first washed with the elution buffer and then equilibrated with the binding buffer. The supernatant from the TEV cleavage was adjusted to 2mM of CaCl₂ and binding buffer was added to 10ml. The calmodulin beads were then added and incubated at 4°C, rotating for 3hrs. The beads were washed 3x with 5 CV of binding buffer and calmodulin binding proteins were eluted by incubating the beads with 3 CV of elution buffer on ice for 15 min. The supernatant was transfer to a clean tube and the elution step was repeated with 2 CV of elution buffer. The eluted samples were combined and precipitated by TCA on ice for 30 min. The samples were centrifuged at 4°C for 15 min., the pellet was washed 3x with acetone and air dried before resuspension in 2x SDS sample buffer. The whole sample was loaded on a 10% SDS gel and after coomassie-blue staining bands were excised and analysed by mass spectrometry. For the SILAC experiment 150x10cm dishes were metabolically labelled for 5 passages with medium containing either ¹²C-Arginine or ¹³C-Arginine. MG132 was applied to ¹³C-Arginine labelled cells for 4 hrs prior to harvesting. Cells were lysed as before and an aliquot was used for determination of protein concentration and western blot analysis. Samples were then mixed in 1:1 ratio and TAP-NEDD8 purification and proteomic analysis was performed as before. Quantitation was performed using the MSQuant software (<http://msquant.sourceforge.net>).

Binding buffer: Buffer A + 2mM CaCl₂.

Elution buffer: Buffer A + 10mM EGTA.

Buffer A: 50mM Tris-HCl pH:8.0, 0.5M NaCl, 1mM $(\text{CH}_3\text{COO})_2\text{Mg}$, 1mM imidazole, 1% NP-40.

MS/MS Analysis

The gel track was excised into approximately 15 sections. Each section was cut into 1mm cubes. These were then subjected to in-gel digestion, using a ProGest Investigator in-gel digestion robot, using standard protocols splitting the gel cubes for each section were split between 2 wells of the 96 well plate if necessary. Briefly, the gel cubes were destained by washing with acetonitrile and subjected to reduction and Σ 10% formic acid and concentrated down to 20 μL (SpeedVac, ThermoSavant). They were then separated using an UltiMate nanoLC (LC Packings, Amsterdam) equipped with a PepMap C18 trap & column. The eluent was sprayed into a Q-Star Pulsar XL tandem mass spectrometer (Applied Biosystems, Foster City, CA) and analysed in Information Dependent Acquisition (IDA) mode. MS/MS data for doubly and triply charged precursor ions was converted to centroid data, without smoothing, using the Mascot Daemon 2.1 (Matrix Science, London) data import filter for Sciex Analyst. The MS/MS data file generated was analysed using the Mascot search engine against MSDB July 2005. The data was searched with tolerances of 0.5 Da for the precursor and fragment ions, trypsin as the cleavage enzyme, one missed cleavage, carbamidomethyl modification of cysteines as a fixed modification and methionine oxidation and GlycineGlycine as a lysine modification selected as variable modifications. The Mascot search results were accepted if a protein hit included at least one peptide with a score above the homology threshold and the MS/MS interpretation accounted for the major peaks.

In vitro de-NEDDylation assay

H1299 cells were transfected with Flag-L11 and His₆-NEDD8 and His₆-NEDDylated proteins were purified as before with the exception of eluting proteins in 5M Gm-Cl, 250mM imidazole, 0.1M Tris pH 6.3. The eluates were rapidly diluted to 1M Gm-Cl by adding 50mM Tris pH 8, 0.005% Tween 80, 2mM reduced glutathione, 0.02mM oxidised glutathione. Refolding was performed by rotating the samples at 4°C overnight. Samples were then dialysed against 50mM Tris pH 8, 0.005% Tween 80, 250mM NaCl. The dialysed samples were then used for a de-NEDDylation assay by adding 1µg of bacterially expressed NEDP1 (Shen et al., 2005) and incubated at 37°C for 5 hrs. Untreated and treated samples were analysed by western blotting with anti-Flag antibody.

Half-life analysis

3x10⁵ H1299 cells in a 6cm petri-dish were transfected with 3µg of Flag-L11, Flag-L5 and NEDP1 or empty pcDNA3 vector as indicated with calcium phosphate. For the ³⁵S-labelling, cells were serum starved for 1hr with Methionine/Cysteine free medium before addition of 350µCi/plate of ³⁵S-Methionine (Amersham). Cells were labelled for 3hrs before washing 2x with PBS and replace with regular RPMI medium. Cells were harvested at the indicated time points (chase period) and cell pellets were frozen. Cell pellets were lysed in 400µl of NP-40 lysis buffer and extracts were precleared with protein G beads (Amersham) before immunoprecipitation with 2µg of anti-FLAG antibody for 3hrs at 4°C. A small aliquot of the extracts was used for western blotting for NEDP1 expression. 50µl of 50% slurry of protein G beads were then added for 1hr at 4°C and immunoprecipitates were then washed 3x with 500µl of NP-40 lysis buffer. 50µl of 2x SDS sample buffer was added, samples were boiled for 5 min. and 30µl of sample was loaded on a 12%

Novex precast gel. The gel was then stained, destained, dried and exposed to X-ray film overnight at -70°C. For cycloheximide (CHX), transfections were performed as above and CHX was added at 30µg/ml in DMSO for the indicated times. Cells were lysed in 2x SDS sample buffer, protein concentration was determined and equal amount of total protein was analysed by western blotting. In both cases signals were quantified using BioRad Quantity One software. Standard deviations were derived from 3 independent experiments.

Ribosome profiling

Ribosome profiling was performed as described in Strezoska et al., 2000. 50µg/ml of CHX was added for 5min before harvesting cells with trypsin. Cells were counted and equal number were lysed in 0.5% NP-40, 130mM KCl, 10mM MgCl₂, 10µg/ml CHX, 0.2mg/ml heparin, 2.5mM DTT, 200 units RNasin (Promega) for 15min. on ice. Samples were centrifuged and supernatant containing 150µg of RNA was loaded on a 10-50% sucrose gradient. Samples were centrifuged for 3hrs at 36,000rpm at 4°C in a SW41 Ti swing out rotor. Ribosome profiling was performed using a density gradient fractionation system (Brandel) with upward displacement and continuous monitoring at 254nm using a UA-6 detector.

Legends to Supplementary figures

S2

H1299 cells were transfected with 2µg of His₆-NEDD8 and 5µg of NEDP1 or pcDNA3 empty vector. Cells were harvested and used for Ni²⁺ pull-down or for total cell extract analysis as before. Ni²⁺ purified eluates were analysed by western blotting with anti-NEDD8 antibody, whereas the total cell extracts with anti-ubiquitin.

S3

Annotated MS/MS spectrum showing ions corresponding to amino acid residues 43-54 of NEDD8. The mass difference between the y6 and y7 ions corresponds to KGG.

S4

H1299 cells were transfected as for the cycloheximide experiment, labelled with ³⁵S-Methionine and lysed as described in Methods. Extracts were used for IPs with anti-Flag antibody or for western blotting with anti-NEDP1 antibody. The control IP was performed with extracts from labelled cells transfected with pcDNA3 vector. Signals were quantitated and presented as percentage difference in intensity. The samples for each set of analysis are from the same blot and the same exposure time was employed.

S5

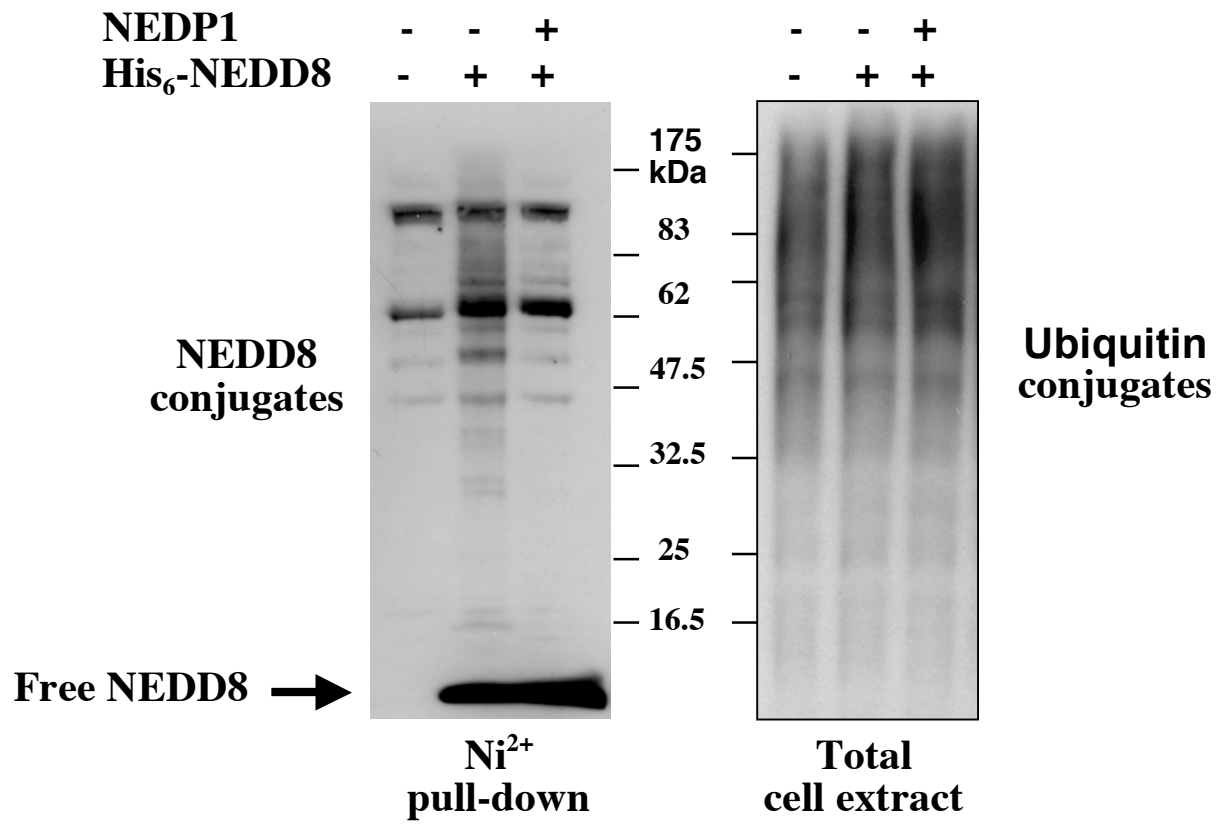
Control CHO or TS-41 cells were grown either at 32°C or shifted to 39°C overnight. Cytoplasmic extracts were isolated and ribosome profiling was performed as described before.

S1

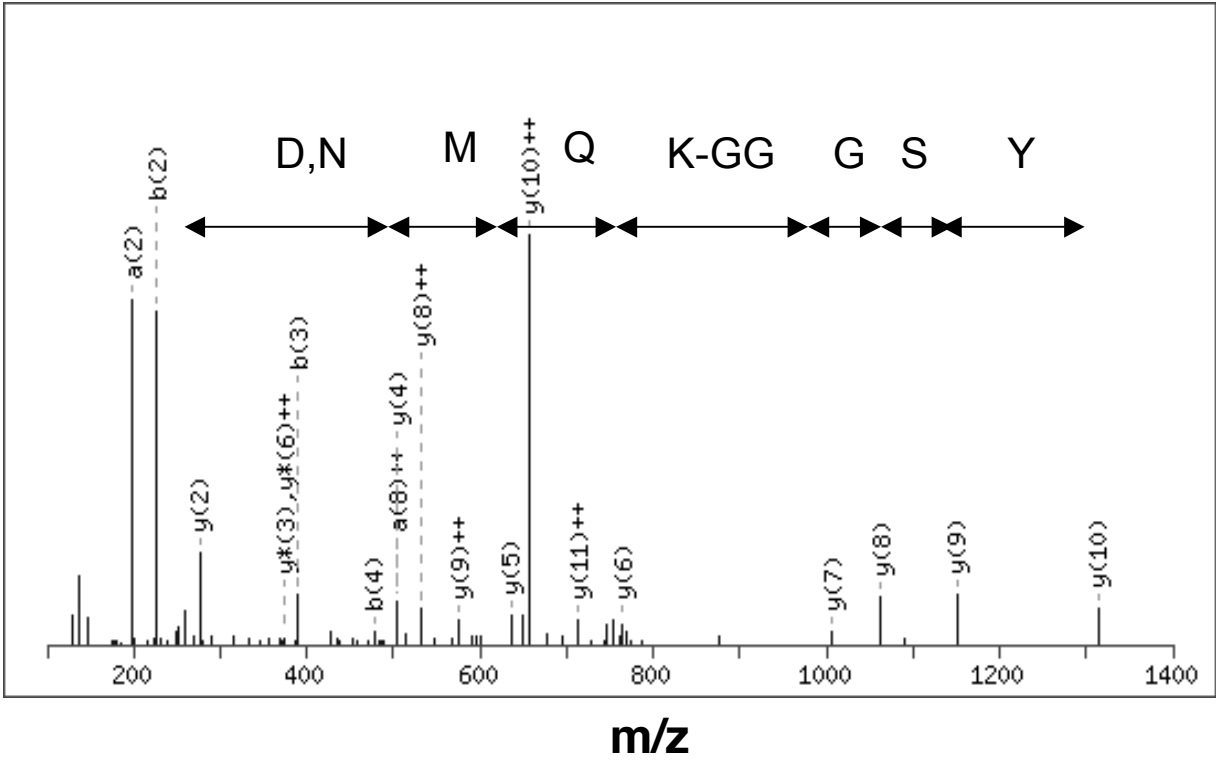
Identified protein	Acc. No.	Mass	Queries matched	Protein score	Seq. cov. %	Peptide score	Pep delta	Peptide sequence
Cullin 4A	gil5565655	88138	55	2976	74	108	0.05	K.FLEETNCLYAAEGQR.L
Cullin 4B	gil55977850	102805	42	2180	55	104	0.04	R.QYQIDAAIVR.I
Protein inhibitor of activated STAT x-alpha (PIASx)	gil3643113	64268	17	1783	34	128	0.08	K.LTADPDEIATTSRLR.V
Cullin 2	gil74744599	87554	44	1736	60	81	0.07	R.KLMVEPLQAILIR.M
Ubiquitin	gil51703339	8560	7	1214	92	104	0.14	K.ESTLHLVLR.L
Elongation factor 2 (EF-2)	gil119172	96115	29	994	34	81	0.09	R.YLAEKYEWDAEAR.K
Cullin 1	gil19863257	90306	37	748	54	118	0.07	R.AGIQQVYTR.Q
Ubiquitin-like molecule NEDD8	gil2833270	9052	6	562	78	79	0.08	K.ILGGSVLHLVLALR.G
Gu/RNA helicase II binding protein	gil7512476	72169	30	561	60	131	0.08	K.LTADPDEIATTSRLR.V
Cullin 3	gil12643396	89444	36	534	50	88	0.1	K.YTFEEIQQETDIPER.E
Cullin 5	gil14917099	91468	22	530	32	69	0.04	K.LALPADSVNIK.I
SMT-3 (SUMO-3)	gil23503102	11607	4	329	37	145	0.09	K.VIGQDSSEIHFK.V
NEDD8-conjugating enzyme Ubc12	gil46577655	21172	10	297	49	65	0.08	R.IQKDINELNLPK.T
Translation elongation factor 1 alpha-2 variant	gil4503475	50780	29	294	62	73	0.06	K.IGGITVPVGR.V
Ribosomal protein L17	gil36126	21611	6	216	36	82	0.07	K.YLKDVTLQK.Q
Ribosomal protein S3	gil417719	26842	14	198	61	88	0.12	R.ELAEDGYSGVEVR.V
Threonyl-tRNA synthetase	gil38202255	84306	31	196	44	60	0.12	R.ILNEKVNTPTTIVYR.C
Eukaryotic translation initiation factor 2A	gil54873624	65590	17	170	32	49	0.04	K.INDFVLSPGPQPYK.V
Ribosomal protein L24	gil4506619	17882	5	169	29	82	0.06	R.AITGASLADIMAK.R
Ribosomal protein S7	gil551251	22127	6	167	37	91	0.04	K.AQQNNVEHK.V
Ribosomal protein L18	gil4506607	21604	5	165	30	53	0.05	K.ILTFDQLALDSPK.G
DNA replication licensing factor MCM4	gil68571766	97068	6	158	8	55	0.09	K.AGICQLNAR.T
14-3-3 protein gamma	gil48428721	28325	5	148	24	83	0.09	K.NVTELNEPLSNEER.N
Ribosomal protein L12	gil4506597	17979	6	147	55	84	0.08	R.CTGGEGVGATSAAPK.I
Voltage-gated sodium channel alpha 1 subunit	gil27263190	226203	10	144	8	52	0.02	K.GWMDIMYAAVDSR.N
Ribosomal protein S6	gil17158044	28842	6	143	28	66	0.06	K.DIPGLTDTTIVPR.R
Histone H2B	gil510991	13606	4	138	35	57	0.06	R.LLLPGELAK.H
Ribosomal protein L11	gil15431290	20167	3	137	20	108	0.09	K.VLEQLTGQTPVFSK.A
Ribosomal protein S8	gil50403622	24475	6	136	38	104	0.12	R.IIDVVYNASNELVLR.T
Ribosomal protein L7a	gil54039239	30148	7	133	31	64	0.1	K.NFGIGQDIQPK.R
Ribosomal protein L8	gil51702823	28235	4	127	19	80	0.14	R.ASGNYATVISHNPETK.K
Ribosomal protein S20	gil46397703	13373	3	125	25	78	0.04	R.LIDLHSPSEIVK.Q
Proliferating cell nuclear antigen (PCNA)	gil129694	29092	6	113	26	73	0.11	K.FSASGELGNGNIK.L
spliceosomal protein SAP 130	gil6006515	135592	23	102	21	43	0.06	R.TVLDPVGTGDLSDTR.T
Ribosomal protein L5	gil81175191	34523	8	99	28	57	0.18	R.VTNRDICQIAYAR.I
Histidyl-tRNA synthetase	gil32460	57410	6	96	14	58	0.02	K.ASAELIEEEVAK.L
Ribosomal protein L30	gil4506631	12947	5	92	51	69	0.06	K.LVILANNCPALR.K
Ribosomal protein L23	gil51338639	15140	6	92	48	70	0.06	R.ISLGLPVGAVINCADNTGAK.N
Rho guanine nucleotide exchange factor 4	gil52782759	76159	12	90	19	42	0.03	K.YPLQLAELLK.Y
Protein inhibitor of activated STAT, 1	gil7706637	71836	7	88	15	47	0.1	K.YCTDCDEIQFK.E
Ribosomal protein L7	gil15431301	29264	12	87	45	56	0.08	R.IALTDNALIAR.S
Lysyl-tRNA synthetase	gil2366752	71795	8	87	15	62	0.1	R.YLDLILNDFVR.Q
Probable ribosome biogenesis protein NEP1	gil20532172	26800	9	85	38	35	0.08	K.VGTSFSIPVSDVRE
Eukaryotic translation elongation factor 1 alpha 1	gil4503471	50451	10	83	21	61	0.08	R.EHALLAYTLGVK.Q
Ribosomal protein S11	gil4506681	18590	9	79	51	48	0.07	K.CPFTGNVSIR.G
Ribosomal protein S23	gil50403755	15838	2	78	15	52	0.07	K.VANLSPVGLALYK.G
DNA repair endonuclease XPF	gil2842712	103288	4	75	5	51	0.12	K.SISDLIGSLNNGR.L
Ribosomal protein L6	gil16753227	32634	4	73	22	38	0.04	K.AVDSQILPK.I
Ribosomal protein S26	gil296452	13336	2	73	17	48	0.05	R.NIVEAAAVR.D
DNA replication licensing factor (huMCM5)	gil12320791	81832	11	67	15	33	0.04	K.SDASPSSIR.S
RNA helicase	gil33150774	54559	17	64	30	27	0.06	K.VLVTTNVCAR.G
Ribosomal protein L27	gil4506623	15788	8	64	53	46	0.06	R.YSVDIPLDK.T
GCN1-like protein 1 (HsGCN1)	gil60390211	213259	4	62	2	38	0.04	K.ALGVMMVK.G
Proteasome 26S subunit, non-ATPase, subunit 2 variant	gil26089272	101322	8	60	12	43	0.04	R.LNILDITLKF
Xeroderma pigmentosum complementation group E protein	gil2632123	126911	12	59	13	25	0.09	R.QQGQQLVTCGAFK.E
Ribosomal protein S4	gil50403628	29807	9	58	35	36	0.16	K.FDTGNLCMVTGGANLGR.I
Ribosomal protein L14	gil7513316	23886	2	54	13	48	0.06	R.ALVDGPCTQVR.R
Ribosomal protein L31	gil4506633	14454	1	53	7	53	0.06	R.SAINEVVTR.E
Ribosomal protein S15a	gil50403624	14813	5	52	30	32	0.04	K.IVVNLTGR.L
Ribosomal protein L21	gil984143	18479	3	50	25	41	0.05	R.VYNVNTQHAVGIVVNK.Q
structure-specific recognition protein, SSRP1	gil730840	73124	4	49	6	36	0.09	K.ADVIAQTGDAICIFR.E
Ribosomal protein L35a	gil22002061	12544	2	49	16	37	0.04	R.DETEFYLGKR.C
Ribosomal protein S16	gil4506691	16549	5	48	30	28	0.05	R.FAGVDIR.V
Ribosomal protein L9	gil15431303	21978	2	47	8	33	0.05	K.GVTLGFR.Y
Ribosomal protein L13	gil15431297	24308	4	47	17	27	0.05	R.TIGISVDPR.R
Ribosomal protein L10a	gil51702773	24856	3	43	17	36	0.16	K.FSVCVLGDQQHCDEAK.A

Histone H2A/x	gil4504253	15135	5	43	35	30	0.07	R.AGLQFPVGR.V
U4/U6-associated RNA splicing factor	gil2853287	77527	10	42	14	31	0.1	K.QLSFISPPTPQPK.T
14-3-3 protein eta	gil1345593	28219	2	42	7	37	0.08	K.NCNDQYESK.V
Amyloid protein-binding protein 1 (APP-BP1)	gil50400302	60665	5	42	15	31	0.04	K.LLCSNSAFLR.V
Ribosomal protein S14	gil5032051	16434	3	41	25	25	0.04	R.IEDVTPIPSDSTR.R
Ribosomal protein L29	gil4506629	17752	2	41	13	28	0.05	K.MQANNAK.A
Ribosomal protein S13	gil4506685	17212	7	40	36	28	0.08	K.GLTPSQIGVILR.D
Ribosomal protein S2	gil1505539	25874	2	37	7	27	0.08	R.GCTATLGNFAK.A
Ribosomal protein L26	gil47117765	17234	2	36	15	28	0.05	K.YVIYIER.V

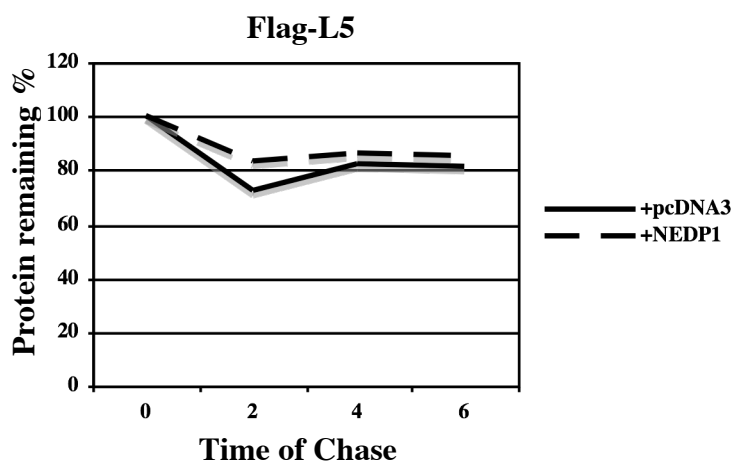
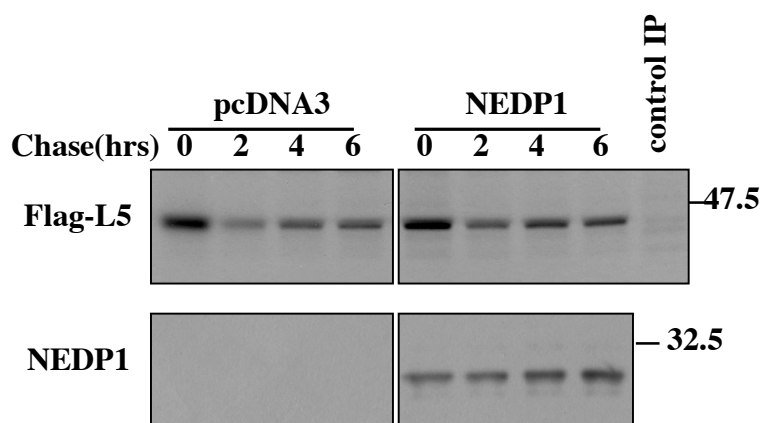
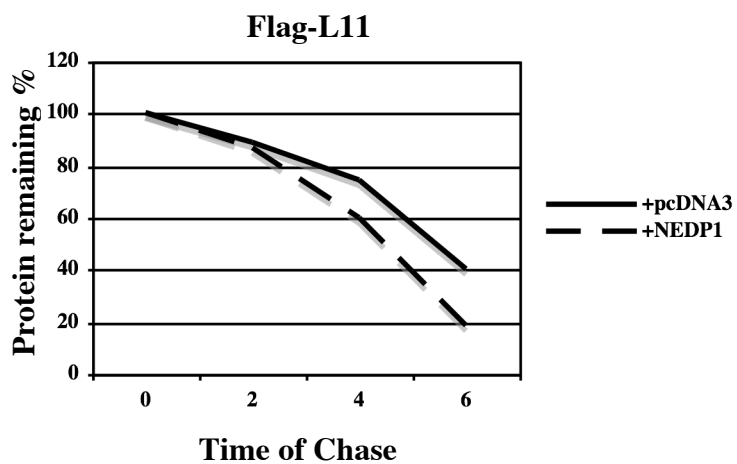
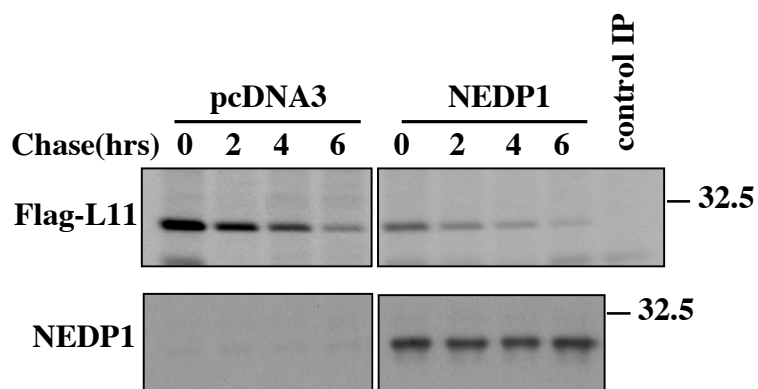
S2



LIYSGKQMNEK
43 48 54
-GG

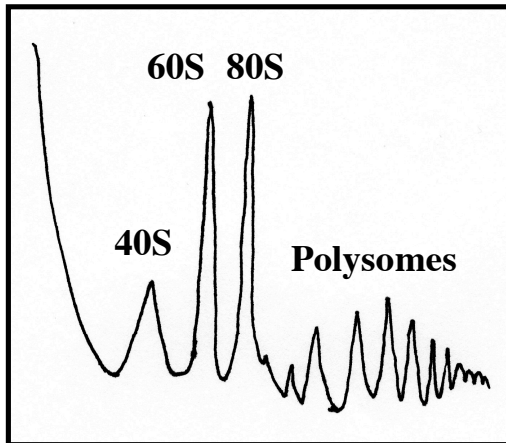


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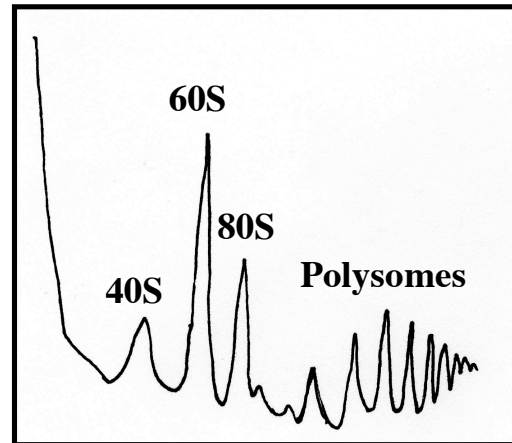


S5

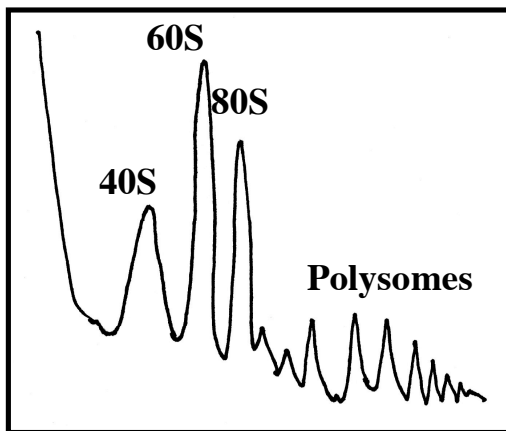
CHO 32°C



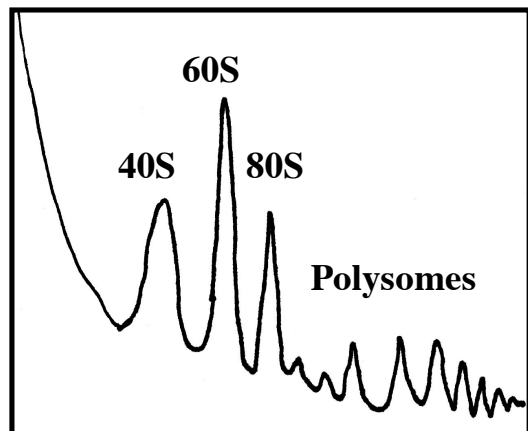
CHO 39°C



TS-41 32°C



TS-41 39°C



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Sedimentation force